Hygroscopicity of Amino Acids and Its Relationship to the Vapor Phase Water Absorption of Proteins[2,3]

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The marked influence of the polar groups upon the vapor phase water absorption of proteins has led to a study of the water absorption of these same polar groups in a number of model substances. In this manner the influence of interactions between polar groups upon the water absorption phenomena could be studied. This paper reports the water absorption results obtained for the amino acids and a number of peptides and other amino acid derivatives. The results indicate that under conditions where the polar groups can be expected to be very highly coördinated into the crystal structure, they absorb little if any water even at high humidities. However, all types of water absorption phenomena, including hysteresis, were shown to be possible within these relatively simple substances. The evidence presented indicates that the polar groups of the proteins must be comparatively uncoördinated and completely available to water molecules. This is a situation similar to that required by the polarization theory of adsorption and, therefore, it is not surprising to find that the polarization theory isotherm describes the water absorption curve of proteins from 6 to 93% relative humidity.

Introduction

The vapor phase water absorption of proteins has been the subject of many investigations and there are still several different explanations propounded for this phenomenon. The coördination of water by polar groups proposed by Lloyd and Phillips[4] has been supported by more direct experimental evidence than any of the other explanations. Sponsler, Bath and Ellis[5] have demonstrated the nature of the binding and the location of the water by infrared absorption and X-ray diffraction measurements on gelatin. Earlier papers in this series[6,7,8] have obtained a more quantitative measure of the absorption due to specific polar groups. Since some of these polar groups are present in the individual amino acids, it has been of considerable interest to determine the hygroscopicity of these amino acids in the pure state and compare the results with the hygroscopicity of peptides and proteins.

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(2) Presented before the 117th Meeting of the American Chemical Society at Philadelphia, Penna., April, 1950.

(3) This is paper VII in a series on the Water Absorption of Proteins.

(4) D. J. Lloyd and H. Phillips, *Trans. Faraday Soc.*, **29**, 132 (1933).

(5) O. L. Sponsler, J. D. Bath and J. W. Ellis, *J. Phys. Chem.*, **44**, 996 (1940).

(6) E. F. Mellon, A. H. Korn and S. R. Hoover, This Journal, **69**, 827 (1947).

(7) E. F. Mellon, A. H. Korn and S. R. Hoover, *ibid.*, **70**, 3040 (1948).

(8) E. F. Mellon, A. H. Korn, B. L. Kokes and S. R. Hoover, *ibid.*, **73**, 1870 (1951).

Experimental

Purification of Amino Acids.—The amino acids were of the purified grade obtainable from regular chemical supply sources. These were used directly as received except for glycine, alanine and arginine hydrochloride which showed traces of hygroscopicity. These three amino acids were recrystallized several times: glycine from water, alanine from 50% alcohol, and arginine hydrochloride from water by the addition of alcohol. Proline, which showed a marked hygroscopicity, was subjected to a more vigorous purification. It was converted to the picrate which was recrystallized twice from water. The picrate was decomposed with 40% sulfuric acid. The picric acid was extracted with ether. The sulfuric acid was removed with barium hydroxide and the water evaporated. The residue was recrystallized twice from hot absolute alcohol.

Preparation of Amino Acid Derivatives.—Hippuryl amide and benzoylglycylglycine were prepared from hippuric acid by the method of Fischer.[9] Ethyl hippurate was prepared from hippuric acid in absolute alcohol by the introduction of anhydrous hydrogen chloride. After removal of

(9) E. Fischer, *Ber.*, **38**, 605 (1905).

the alcohol and acid by vacuum evaporation and treatment with potassium bicarbonate, the product was recrystallized twice from water.

The glycine methyl and ethyl ester hydrochlorides were prepared from glycine and the corresponding alcohol by the addition of anhydrous hydrogen chloride and the products were recrystallized from absolute alcohol.

Glycylglycine hydrochloride was made from glycine anhydride and concentrated hydrochloric acid and was recrystallized twice from 95% alcohol.

Carbobenzoxyglycine was prepared according to Carter.[10] Carbobenzoxyglycylglycine was prepared according to Bergmann and Zervas,[11] and carbobenzoxytriglycine was prepared according to Bergmann, Zervas and Fruton.[12]

Allylglycylglycine was made from 57 g. of glycine anhydride which was dissolved in 500 ml. of 2 *N* sodium hydroxide for one-half hour at room temperature and then added in 40-ml. quantities to a vigorously stirred ice-cold solution of 125 g. of α-bromopropionyl bromide in 400 ml. of dry chloroform. Additional alkali was added as required to keep the mixture basic. After 15 minutes the chloroform layer was separated and the water layer was neutralized with concentrated hydrochloric acid and evaporated to 400 ml. On cooling, crystals separated which were filtered and recrystallized from hot water. Eighty grams of this α-bromopropionylglycylglycine was dissolved in 600 ml. of 9 *N* ammonium hydroxide and heated on a water-bath at 50° for 4 hours. The solution was evaporated to dryness *in vacuo*. The residue was dehydrated with absolute alcohol and the crystals separated. These were recrystallized twice from a small amount of water by adding a large amount of absolute alcohol to yield 46 g. of alanylglycylglycine monohydrate, m.p. dec. 207°. The analysis of the anhydrous material was N, 20.43; NH_2-N, 6.8% (theory N, 20.68; NH_2-N, 6.9).

The Absorption Determination.—About 2-g. quantities of these materials were dried under a 29″ vacuum at 70° with a stream of dry air flowing through the oven to remove the liberated moisture.[6] The samples were dry within 14 hours. A subsequent drying period of 6 hours demonstrated that an equilibrium dry weight had been attained. The dried samples were put into desiccators over saturated salt solutions, and after evacuation to about 40 mm. pressure, the desiccators were submerged in a water-bath the temperature of which was regulated to 30.0 ± 0.1°. The samples were weighed every 3 days until equilibrium was demonstrated. A weight change of less than 1 mg. between consecutive weighings was indicative that equilibrium had been attained. After equilibrium was established at one humidity the samples were then exposed to the next desired humidity and the weighing procedure repeated. After the humidity studies were completed on a sample, it was redried to demonstrate the constancy of the dry weight. The salt solutions used and other details of the procedure are described fully in the first paper of the series.[6]

Limits of Hygroscopicity.—The problem of determining the boundary line between hygroscopic and non-hygroscopic materials depends to a large extent upon the refinements of the methods of detecting absorbed moisture and the purpose of the researcher. We have not been interested in the extremely small amounts of moisture necessary to form a few layers on a crystal surface and have limited our study to amounts which are weighable on the average analytical balance. With a 2-g. sample, an increase in weight of 2 mg. was found to be significant. The materials which we list as non-hygroscopic do not show weight changes of this magnitude except for the few cases for which a specific absorption is given. In these few exceptions the absorption is slight and can probably be attributed to impurities in these samples which have not been exhaustively purified.

Results

Non-hygroscopic Amino Acids.—Except for the few cases where exact absorptions are recorded, the following amino acids are non-hygroscopic at all humidities up to and including 93% relative humidity: DL-alanine, DL- and L-aspartic acid, L-cystine (0.8%), L-glutamic acid, glycine, hydroxy-L-proline (0.5%), DL-isoleucine, DL- and L-leucine, DL- and L-methionine, DL-norleucine, DL-phenylalanine, DL-serine, L-tyrosine and DL-valine.

Non-hygroscopic Amino Acid Derivatives.—Among the amino acid derivatives and peptides studied the following were found to be non-hygroscopic at all humidities up to and including 93% relative humidity: L-proline picrate, glycine anhydride, carbobenzoxyglycine, benzoylglycine, benzoylglycine amide, ethyl hippurate, glycylglycine and benzoylglycylglycine.

Hygroscopic Amino Acids and Derivatives.—L-Lysine monohydrochloride is apparently non-hygroscopic at 51% relative humidity but shows an absorption of exactly two moles of water at 75% relative humidity. This hydrate persists at 83% relative humidity but on standing for 10 days at this humidity, moisture begins to condense on the sides of the weighing bottles and the samples dissolve. We did not make any absorption determinations between 51 and 75% relative humidity so we are unable to say whether there are two steps to the formation of this dihydrate or to give the exact humidity of the transition point. This latter point is also true for the remaining compounds to be discussed in this section but the limits assigned to the transition region are sufficient for the purposes of the discussion.

L-Proline does not absorb moisture at 31% relative humidity but forms a monohydrate at 51% relative humidity. This hydrate will dissolve on standing at 51% relative humidity but will revert to the anhydrous condition when the humidity is lowered to 31% again.

L-Asparagine does not absorb moisture at 75% relative humidity but absorbs 1 molecule of water at 93% relative humidity. This transition requires a considerable length of time to attain equilibrium.

The hydrochlorides of the methyl and ethyl esters of glycine both remain non-hygroscopic at 51% relative humidity but dissolve completely in the water which they absorb at 75% relative humidity.

Glycylglycine hydrochloride crystallizes from 95% alcohol as a monohydrate. This hydrate will lose water slowly at 70° or at 30° in low humidities. At 105° the water is lost rapidly to give the anhydrous material. Material which is partially dried at 70° or at low humidities will dissolve in its absorbed water at 75% relative humidity while the material which has been thoroughly dried remains dry at 83% relative humidity and dissolves in its absorbed water at 93% relative humidity. These phenomena are summarized in Fig. 1.

Alanylglycylglycine has given an absorption isotherm (Fig. 2) which indicates a hydrate formation but the transitions from the anhydrous material to the mono- and dihydrate states are not very sharp. Moreover, this material remains as a dihydrate on desorption even after the humidity has been reduced to 6% relative humidity.

Carbobenzoxyglycylglycine absorbs only slightly at 51% relative humidity and attains an absorption of 0.89 mole per mole at 75% relative humidity. This probably indicates the formation of a mono-

(10) H. E. Carter, *Org. Syntheses*, **23**, 13 (1943).

(11) M. Bergmann and L. Zervas, *Ber.*, **65**, 1200 (1932).

(12) M. Bergmann, L. Zervas and J. S. Fruton, *J. Biol. Chem.*, 111, 237 (1935).

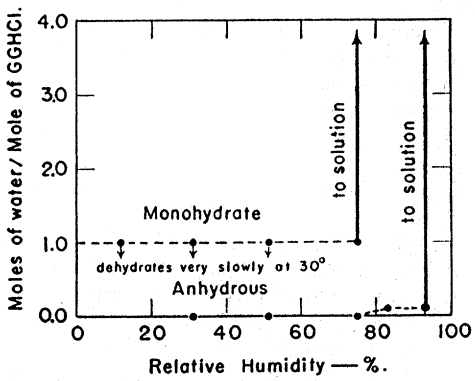

Fig. 1.—Water absorption of glycylglycine hydrochloride.

hydrate and the presence of 10–15% of a non-hygroscopic material as an impurity.

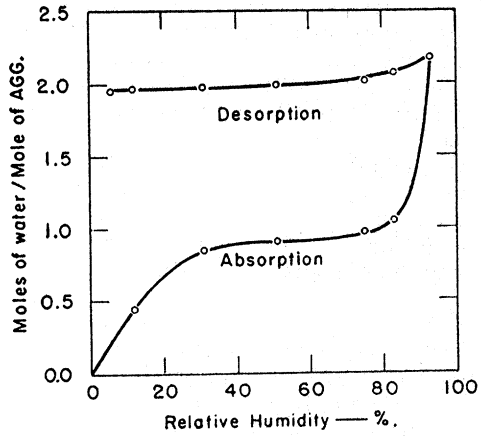

Fig. 2.—Water absorption of alanylglycylglycine.

Figure 3 shows that L-arginine hydrochloride and carbobenzoxytriglycine exhibit no tendency to hydrate formation but do have an appreciable absorption at the higher humidities.

Discussion

The lack of hygroscopic tendencies in certain of the pure amino acids and their simple derivatives indicates that the polar groups which are present in these compounds must be coördinated in the crystal structure in such a way that they are unable to coördinate additional water molecules or the crystals must be so compact that water is unable to penetrate into them. Frey and Moore[13] have shown that glycine, leucine and glycine anhydride do absorb water vapor upon their surface in about the same molar proportions as the polar groups are present in these surfaces. The amount of water adsorbed in this manner is very slight (about 2×10^{-5} mole/gram at 90% RH) when compared on a total weight basis, but it is sufficient to show that these polar groups would absorb water if they were free to do so. Their results also show that the adsorption of the water on these surface polar groups follows a sigmoidal isotherm and is not a stepwise increase with increasing humidity. These results indicate that wherever a polar group is not strongly coördinated to neighboring groups its absorption

(13) H. J. Frey and W. J. Moore, This Journal, **70**, 3644 (1948).

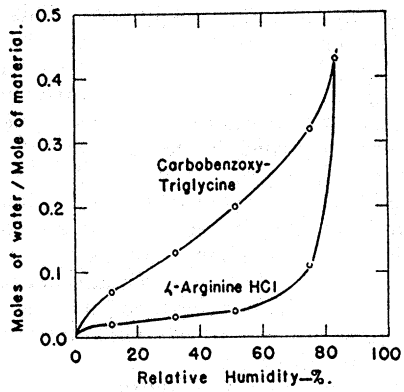

Fig. 3.—Water absorption of L-arginine hydrochloride and carbobenzoxytriglycine.

follows a mass action phenomenon where the amount absorbed is a function of the pressure of the water vapor present. Carbobenzoxytriglycine and L-arginine hydrochloride (Fig. 3) are some of the crystalline model compounds which show that this type of absorption may occur within the crystal lattice.

The similarity of the absorption isotherms for these surface polar groups of crystals and the polar groups of proteins indicate that the polar groups of the proteins must be comparatively uncoördinated and free to absorb water vapor. This has already been demonstrated[14] to some extent by the lack of dependence of water absorption upon the physical structure of a variety of proteins.

With some materials (L-lysine monohydrochloride, L-proline and L-asparagine) the crystal structure appears to be capable of a transformation or phase change to accommodate a definite number of water molecules per mole of material. These transformations occur over a very short range of relative humidity and the absorption curve proceeds in a stepwise fashion. There has been no evidence that this type of absorption occurs to any extent in proteins.

The formation of a hydrate seems to be a step in the opening of a crystal structure to water vapor because most of the amino acids and derivatives which have formed hydrates show evidence of subsequent hydration which in most cases leads to solution of the material. Glycylglycine hydrochloride gives an excellent example of this effect. The anhydrous material remains anhydrous (Fig. 1) until it dissolves at a humidity between 83 and 93%. The material which has been crystallized with one molecule of water of crystallization will dissolve at a humidity between 51 and 75%. The hydrated crystal, therefore, has a greater hygroscopic tendency than the anhydrous crystals. This behavior should cause a hysteresis type of phenomenon for on desorption a solution of the anhydrous material would be expected to produce the monohydrate as an intermediate step and this monohydrate should persist at lower humidities where the anhydrous material remains unhydrated on the absorption cycle.

A more striking demonstration of a hysteresis phenomenon and one which begins to approach the

(14) E. F. Mellon, A. H. Korn and S. R. Hoover, *ibid.*, **71**, 2761 (1949).

type of absorption isotherm found in proteins is exhibited by alanylglycylglycine. This material absorbs water with increasing relative humidity to give a sigmoidal isotherm (Fig. 2). The center portion of this isotherm is comparatively flat and indicates an absorption of nearly one molecule of water per mole, which is the water of crystallization present in the material as recrystallized from water by the addition of absolute alcohol. This behavior would indicate that we do not have a crystal transformation or phase change during this early stage of the absorption but only a mass action type of equilibrium in which water is coördinated on a polar group which appears to be available to coördinate one molecule of water.

The second step in this hydration appears to be a crystal transformation since slightly more than one molecule of water is absorbed for a slight change in relative humidity. This transformation is further substantiated by the fact that on desorption this material remains as a dihydrate even at 6% relative humidity. A definite hysteresis has, therefore, been demonstrated which appears to be due to a structural change in the crystal lattice.

Many careful investigators have been convinced that the experimental data they secured showed a definitely higher amount of water absorbed by proteins when the equilibrium was approached from the "wet" side than when it was approached from the "dry" side. This phenomenon, called hysteresis, was confirmed in an earlier study[15] of sorption by a group of benzoylated casein samples. A marked hysteresis was observed and graphical analysis of the data showed that it was of two types, a portion dependent on the relative humidity from which the desorption was started, and a second portion which was of constant magnitude and persisted undiminished as the humidity was lowered to 6% relative humidity. Our model compound, alanylglycylglycine, exhibits a similar hysteresis behavior; for the dihydrate which is formed only at the higher humidities persists on desorption to somewhere below 6% relative humidity. This is the first experimental evidence of which we are aware that sorption hysteresis of proteins may be ascribed to a thermodynamically satisfactory hydrate transition of the type clearly shown by alanylglycylglycine.

(15) E. F. Mellon, A. H. Korn and S. R. Hoover, *ibid.*, **70**, 1144 (1948).

Urquhart[16] postulated such an explanation of hysteresis many years ago, and White and Eyring[17] have discussed it similarly in terms of the theory of absolute reaction rates. The latter workers concluded that "sorption hysteresis is a mechanical lag in the response of the network structure caused by the strong adsorption of molecules on geometrically important sites." This seems to describe the water absorption phenomena of alanylglycylglycine very adequately.

The differences in behavior between benzoylglycylglycine, which is non-hygroscopic, carbobenzoxyglycylglycine, which forms a hydrate, and carbobenzoxytriglycine, which gives a sigmoidal absorption isotherm, show the variety of water absorption phenomena in the realm of purely crystalline materials even when they are of closely related chemical structure. It is obvious that the effect of polar groups upon water absorption depends considerably upon how these groups are coördinated in the molecular structure. The water absorbing capacity of a polar group in a highly coördinated crystalline compound should, therefore, not be used to describe the effect of the same polar group when it is in a comparatively poorly coordinated structure like a protein.

One should remember that although the polar groups of a protein may be holding the protein chains together by electrostatic forces these same polar groups may be completely available for the coördination of small dipolar molecules such as water. This situation is similar to the requirements of the polarization theory of the absorption of dipolar gases upon polar groups as proposed by Bradley.[18] Moreover, this polarization isotherm has been shown[19] to describe the vapor phase water absorption of proteins and other high polymers over 85% of the entire relative humidity range.

The series—amino acids, peptides and proteins—therefore, appears to demonstrate very nicely the transition in vapor phase water absorption phenomena from purely crystalline phase changes to strictly dipole interactions.

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(16) A. R. Urquhart, *J. Textile Inst.*, **20**, T125 (1929).

(17) H. J. White, Jr., and H. Eyring, *Textile Res. J.*, **17**, 523 (1947).

(18) R. S. Bradley, *J. Chem. Soc.*, 1467, 1799 (1936).

(19) S. R. Hoover and E. F. Mellon, THIS JOURNAL, **72**, 2562 (1950).